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Phytochemicals enriched *Punica granatum* L. peel extract promotes the chondrocyte proliferation by inducing cell cycle progression and inhibiting of NO-induced cell death and ROS production

Neelam Shivnath[a,*,†], Vineeta Rawat[a], Sahabjada Siddiqui[b], Asif Jafri[a], Juhi Rais[a], Madhu Gupta[a], Mohd Sajid Khan[c,d] & Md Arshad[a,e,*,‡]

[a]Molecular Endocrinology Lab, Department of Zoology, University of Lucknow, Lucknow 226 007, Uttar Pradesh, India

[b]Department of Biotechnology, Era's Lucknow Medical College & Hospital, Era University, Lucknow 226 003, Uttar Pradesh, India

[c]Department of Biosciences, Integral University, Lucknow 226 026, Uttar Pradesh, India

[d]Department of Biochemistry, [e]Department of Zoology, Aligarh Muslim University, Aligarh 202 002, Uttar Pradesh, India

E-mail: [†]neelamshivnath@yahoo.co.in; [‡]arshadm123@rediffmail.com

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Punica granatum L. has been used as a traditional remedy to treat sore throat, cough, digestive and skin disorders, urinary infection, arthritis and expel tape-worm. Modern research focuses on its use on arthritis, diabetes and cancer. The present study was conducted to identify the bioactive phytochemicals in the ethanolic extract of *Punica granatum* peel (PGP) through GC-MS to illustrate its proliferation, antioxidant and anti-apoptotic activities in an established *in vitro* primary cultured chondrocyte. The cells were treated with 25 µM indomethacin, positive control and different concentration of PGP in the absence or presence of different inhibitors such as (H_2O_2 for ROS production and sodium nitroprusside [SNP] for nitric oxide [NO] induced cell death). The cell viability assay and cell cycle analysis were performed to study the proliferation of cells. Oxidative stress was measured as intracellular ROS and anti-apoptotic activities were studied through nuclear staining assays like DAPI and PI-Exclusion staining. The GC-MS result indicates the presence various antioxidants such as kaempferol, quercetin, myricetin, luteolin, ascorbic acid, gallic acid, vitamin C and lycopene. The PGP treatment on chondrocytes shows increased proliferation and decreased apoptosis through the reduction of oxidative stress in a concentration-dependent manner. The rise in the total number of chondrocytes at higher doses of PGP was significant ($p<0.05$) as compared to indomethacin (positive control) treated cells. The findings suggest that PGP exhibits therapeutic antioxidant potential due to the presence of bioactive components regulating oxidative stress , promoting the chondrocytes proliferation and inhibiting apoptosis of chondrocytes.

Keywords: Apoptosis, Chondrocytes, Osteoarthritis, Oxidative stress, Phytochemicals

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Osteoarthritis (OA) is a serious joint disorder, which is characterized by slow degradation of articular cartilage that leads to chronic pain and joint inflammation[1-3]. The mature cartilage consists of only chondrocyte cells, which is responsible for maintaining the entire integrity of the extracellular matrix (ECM) and keeps regulating dynamic equilibrium between formation and degradation of ECM[4,5]. The chondrocytes are incapable of self-renewal because they are dormant, therefore, chondrocyte proliferation and death play an important role in OA pathogenesis in which oxidative stress plays a significant role in the apoptosis. The key components released during the course of the disease are reactive oxygen species (ROS) and nitric oxide (NO)[6]. NO is a free radical produced by nitric oxide synthase (NOS) that enhances joint vasodilation and permeability by increasing TNF-β and IL-1α production[7]. NO acts by inhibiting the synthesis of collagen and proteoglycan which in turn stimulates matrix metalloproteinases (MMPs) inducing the apoptosis of chondrocytes[6]. Intracellular ROS is generated during a cellular metabolic process. The proliferation and maturation of chondrocytes are associated with the formation of ROS. The proliferating chondrocytes show high proliferation activity through induction of nox1 expression that is responsible for ROS production[8]. Further, during the maturation of chondrocytes, the activity of the catalase enzyme decreases that further increasing the

*Corresponding authors

ROS production in chondrocytes[9]. Overproduction of ROS may inhibit the proliferation in late-proliferating chondrocytes in mature cartilage. The reactive oxygen species have been involved in cellular damage. Oxidative stress occurs due to cell death releases the cellular content into the extracellular environment that leads to synovial inflammation. Chondrocytes contain the number of an antioxidant defense system against ROS and failure of these systems leads to degeneration of articular cartilage[10]. The harmful effects caused due to oxidative stress may be rescued by antioxidant treatment.

Among the pharmacological treatments, non-steroidal anti-inflammatory drugs (NSAIDs) have been used in the management of OA but have been associated with serious side effects of gastrointestinal bleeding, renal and cardiovascular diseases[11,12]. Importantly, there is a requirement for safe and effective treatment developed from natural therapeutic obtained from plants with no or reduced side effects. Dietary polyphenols have a ROS scavenging potential, which helps to inhibit ROS-generating enzymes and improves the production of antioxidant enzymes[13].

Punica granatum L. (Lythraceae), one of the oldest fruits with medicinal properties has long been used to treat a multitude of disease conditions including chronic pain[14,15]. *P. granatum* has been used as a traditional remedy to treat sore throat, cough, digestive and skin disorders, urinary infection, arthritis and expel tape-worm. Modern research focuses its use on arthritis, diabetes and cancer[16]. This plant is a native of Iran and has been cultivated in Egypt, India, Bangladesh, Sri Lanka, North Africa, California and Arizona[17]. The fruit is covered by a pericarp and widely used for multiple therapeutic purposes[18]. The previous studies on the preliminary phytochemical screening of the ethanolic extract of *P. granatum* peels (PGP) gave positive tests for flavonoids, flavones, terpenoids, steroids and tannins showed that the ethanolic extract of PGP may have some biologically active components[19,20]. The studies have revealed that hydro alcoholic peel extract of pomegranate improves the clinical symptoms associated with knee OA in double-blind placebo control study[21].

In the present work, we studied the bioactive phytochemicals of PGP that have therapeutic antioxidant potential and assessed if the group of active compounds in the extract might inhibit the important molecules like ROS and NO that are responsible for the degeneration of chondrocytes. For the study, *in vitro* oxidative stress model has been developed and the mechanistic roles of PGP on chondrocyte were studied through cells proliferation, cell cycle analysis and inhibition of ROS and nitric oxide (NO) induced cell death.

Materials and Methods

Chemical and reagents

Dulbecco's modified eagle medium/nutrient mixture F-12 Ham's (DMEM/F-12), sodium pyruvate, non-essential amino acids, fetal bovine serum (FBS), sodium bicarbonate, MTT (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) dye, antibiotic (penicillin/streptomycin), trypsin/ EDTA (0.25%), collagenase type II, were procured from Himedia, India. Sodium nitroprusside, dimethyl sulphoxide (DMSO), 2',7'-dichlorofluorescein diacetate (DCF-DA) dye, 4',6-diamidino-2-phenylinole (DAPI) staining were purchased from Merck, India.

Collection of plant material

Pomegranate fruits were collected from a market, Aminabad, Lucknow, India and identified by Dr. S. Lavania, Head, Botany Department, Lucknow, University, Lucknow. The specimen's voucher number (Voucher ID. LWU- 2016-3) was submitted in the herbarium, Botany Department, Lucknow University, Lucknow.

Preparation of plant extract

From the fresh fruits of *Punica*, the peel was separated and washed two times with double distilled water and dried it under shade. They were turned into powder using a mixer grinder. The 95% ethanolic extract of *P. granatum* peel (PGP) was prepared using the Soxhlet apparatus (Borosil Glass Works Limited, India). The obtained filtrate was concentrated by heating it over the mantle to yield 10% of the final filtrate[22]. The phytoconstituents of the extract were analyzed using GC-MS and used for *in vitro* studies to evaluate its chondrogenic efficacy.

GC-MS analysis

The sample (100 µL) was placed into an eppendorf tube following the addition of methoxyamine hydrochloride (80 µL) and N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) (100 µL). The samples were kept for 1 h at 65^{o}C in thermoshaker (Thermoshaker Incubator, BRTH- 100; BR Biochem Life Science Pvt. Ltd.). The derivatized phase

obtained after filtration was transferred into GCMS/MS vial. The identification of distinct components of an ethanolic fraction was done using a GCMS/MS (Thermo Scientific TSQ Quantum XLS, TriPlus autosampler with a triple quadrupole). On a DB- 5MS (30 m x 0.25 mm I.D. with 0.25 m thickness of film) column, the sample (1 µL) was injected. Helium gas (99.9%) was used as the carrier gas and was supplied with a constant rate of flow (1.2 mL/min). The injector port was maintained at 250°C, the mass transmission line was at 300°C and the oven at 65°C (for 2 min). The solvent delay time was set at 6.5 min and the total time the GC-MS operated in full scan mode was kept at 55 min. Using mass spectral research library (NIST), the mass fragmentation pattern of several substances produced (by electron ionization) was identified based on their retention duration.

Ethics statement

Animal experiments were implemented using the standard protocol of the Animal Ethics Committee of the Institution (IAEC), Pharmacy Department, Integral University, Lucknow. The approval number of this study was IU/Biotech/Project/ IAEC /15 /17.

Primary cell culture of chondrocytes

The chondrocytes for primary culture were harvested from the knees of rat pups (2-3 days old). The cartilages were placed into a Petridish containing phosphate buffer saline (PBS) and antibiotics. The cartilage was cut into small fragments and digested with trypsin/EDTA (0.25%) for 30 min and subsequently digested two times with 0.2% collagenase type II (125 units/mg) in DMEM/F-12, for 1 h. The cells were collected after brief centrifugation and resuspended in DMEM/F-12 media with 10% fetal bovine serum (FBS) and antibiotic solution. They were cultured at a density of 3×10^4 cell/cm^2 of culture flask. Media was replaced after every two days and reached confluency at ~5 days of culture[23].

Cell proliferation assay

At passage one, chondrocytes were seeded at a density of 1×10^4 per well into a 96- well plate and left overnight for incubation in the CO_2 incubator. The cells were transferred to serum-free media for 24 h and exposed to various concentrations of PGP extract (25, 50, 100, 250 and 500 µg/mL). The control was given no treatment and positive control (PC) cells were treated with Indomethacin (IM at 25 µM). The effective dose (ED_{50}, 25 µM) of IM was calculated based on MTT assay on primary chondrocytes (graph not given) and it was used as a positive control in all studies. After 24 h of treatment, the media from each well was aspirated and 20 µL of MTT (stock solution of 5 mg/mL) added to each well and incubated for 3 h. Further, 50 µL/well DMSO was added to each well, and absorbance was taken at a wavelength of 520 nm with ELISA plate reader[24]. The minimum effective doses of PGP were found to be 50, 100 and 250 µg/mL, therefore, these three doses were considered in further studies. The % cell proliferation was calculated by the formula:

$$\% \text{ Cell Proliferation} = [(\text{OD of treated})/(\text{OD of control})] \times 100$$

Cell cycle analysis

The second passage of chondrocytes was seeded at density 1×10^4 into a 6-well plate and cultured for 24 h. The next day, the cells were transferred to serum-free media for 24 h. The cells were treated at 50, 100, 250 µg/mL of PGP extract and IM (25 µM) and further incubated for 24 h. The cells were fixed with 75% cold ethanol and then incubated overnight at 4°C. Afterwards, RNase A (100 mg/mL) was added and cells were stained with propidium iodide (PI) for flow cytometric analysis[23].

ROS production

The dye 2',7'-dichlorofluorescein diacetate (DCF-DA) was used to detect ROS generation. Chondrocytes were implanted in a culture plate with a black base (37°C, 5% CO_2) for 24 h. Except for control, the cells were subjected to a 20 µL H_2O_2 solution (10 M stock) for 24 h. The PC group was treated with IM, 25 µM in triplicate and incubated for 24 h after being exposed to various doses of PGP (50, 100, 250 µg/mL). After the incubation time, the cells are stained for 30 min at room temperature in the dark with DCFH-DA dye (stock 10 mM). At a wavelength of 528 nm, fluorescence was measured using a Microplate reader (Synergy H 1 Hybrid Multi-Mode and Absorbance Reader, Agilent). A fluorescent microscope (Eclipse Ti- S, Nikon, Japan) was used to photograph another batch of cells seeded in a 96 well plate to investigate the fluorescence intensity of intracellular ROS production[25].

MTT assay for nitric oxide (NO) induced cell death

Chondrocytes were grown at 1×10^4 cells/mL of media in 96-well plate for a day at 37°C in 5% CO_2

and 95% humidified air. The seeded chondrocytes were pretreated with IM (25 μM) and varying concentrations (50, 100, 250 μg/ mL) of PGP for 24 h. Cell death was stimulated by exposure of cells to 1.5 mM of sodium nitroprusside (SNP) for 24 h[26]. After that, cell viability was determined using the MTT dye.

Nuclear condensation assay

Under UV light, 4', 6-diamidino-2-phenylinole (DAPI) staining binds to dsDNA and produces a blue fluorescence. In multiwell plate, seeded cells were pre-treated with PGP (50, 100, 250 μg/mL) and IM for 24 h before exposure to SNP. The cells were rinsed twice with PBS and subsequently fixed with 4% PFA, 10 min following 24-h of SNP treatment. The cells were then stained with DAPI after being permeabilized using permeabilization buffer (3.0% PFA and 0.5% Triton X-100). A fluorescent microscope (Eclipse Ti- S, Nikon, Japan) was used to capture the photographs[27].

Propidium Iodide (PI) staining for apoptosis

This stain is used for selective labeling of cells in late apoptotic phase. This dye does not penetrate intact cell membranes, but may freely enter cells with a compromised cell membrane. After the treatment with SNP, the monolayer is washed three times with PBS and fixed in 4% paraformaldehyde (PFA). The cells were washed with 100 μL of PBS and 1 μL of PI dye (5 mg/mL) was added into each well and incubated for 10 min. The cells were washed with PBS, stained with 1 μL of PI dye (5 mg/mL) and incubated for 10 min. Morphological changes in cells were observed under the fluorescence microscope (Nikon, ECLIPSE Ti-Series).

Statistical analysis

All statistical data was assessed by GraphPad Prism version 0.7 software (GraphPad prism software Inc) and calculated as mean ± SEM. Statistical analysis of *in vitro* were analyzed by one-way ANOVA and P values less than 0.05 ($p<0.05$) were considered as statistically significant.

Results

GC-MS analysis

The GC-MS analysis of the ethanolic peel extract of *P. granatum* shows about 34 peaks representing the presence of a total of 34 bioactive compounds. The PGP demonstrates the presence of a rich source of bioactive phenolic components including flavonoids (quercetin, kaempferol, myricetin, luteolin), á-sitosterol, gallic acid, coumarins, vitamin C and lycopene. Apart, a large number of sugars mainly glucose, fructose, glucitol etc., Vit C, D3 metabolite and polyunsaturated fatty acids are also present. Each component present in the GC-MS chromatogram has been interpreted by the assessment of their % peak area with retention time and compared with the mass spectra from a data library as mentioned in Table 1. The mass spectra of the different compound were shown in Fig. 1.

Primary culture of chondrocytes

The chondrocytes adhere to culture dishes and become confluent forming monolayer in 5-6 days. The chondrocytes in primary culture are small polygonal cells and regarded as passage 1 chondrocytes. The chondrocytes at passage 2 become spindle shape more like fibroblast cells and the cells at passage 2 grow faster than cells at passage 1. After 4-5 passages, the growth rate slows shape changes to oval and start detaching from the surface of the flask (Fig. 2).

Effect of ethanolic peel extract and indomethacin (IM) on chondrocytes proliferation

Treatment of cells with PGP at various doses (25-500 μg/mL) considerably increases the absorbance value in a concentration-dependent manner as compared to the control. The greatest value of OD was found at a higher concentration (500 g/mL). As cells were exposed to IM at a concentration of 25 μM, their proliferation increased by 159.54% when compared to control. The proliferation of cells exposed to PGP at doses of 25, 50 and 100 μg/mL was 125.6, 141.34 and 159.2%, respectively. The significant increase in cell proliferation was observed at 250 μg/mL which was about 2 folds compared to control and more than two folds at 500 μg/mL of PGP. The proliferation of cells reduces with a further increase in the concentration of dose above 500 μg/mL and there is a significant reduction in chondrocytes number. Therefore, the three effective doses 50, 100 and 250 μg/mL were included for further studies. The rate of absorbance was proportional to the chondrocytes number, which was enhanced significantly at higher doses of PGP (Fig. 3).

Determination of cell proliferation by flow cytometry

The cell cycle analysis was used to measure the amount of DNA in the cells during the different phase of cell cycle. Compared to the control group, the mean proportion of cells in S phase rose from 13.0 to 18 % in the IM, whereas 16.2, 19.99 and 27.73% in

Table 1 — The composition of bioactive phytochemicals present in ethanolic extract of *Punica granatum* peel screening through GC-MS:

S. No.	Compound Name	MF	MW	RT	% Peak Area	Nature
1	Propanedioic acid, ethyl-, diethyl ester (CAS)	$C_9H_{16}O_4$	188.220	7.73	0.93	Carboxylic acid, ester
2	cis-5,8,11-Eicosatrienoic acid, methyl ester	C21H36O2	320.509	320.509	0.05	Polyunsaturated ω-6 fatty acid
3	9,12-Octadecadienoic acid (Z,Z)-	C18H32O2	280.445	11.61	0.21	Linoleic acid (polyunsaturated ω-3fatty acid)
4	Trimethylsilyl ether of glycerol	C12H32O3Si3	308.637	12.77	0.25	Glycerol ether
5	9,12,15-Octadecadienoic acid, (Z,Z,Z)-	C18H30O2	278.429	14.05	0.01	Mono unsaturated ω-9 fatty acid
6	Tristrimethylsilyl ether derivative of 1,25-Dihydroxyvitamin D2	C37H68O3Si3	644	16.66	0.12	Vit D3 metabolite
7	2,3,4-Trihydroxybenzoic acid	C7H6O5	170.119	17.75	2.04	Pyrogallol (carboxylic acid)
8	Vitamin C	$C_6H_8O_6$	176.124	18.85	0.12	Ascorbic acid
9	D-(+)-Xylose, tetramethyl ether	$C_9H1_8O_5$	206.236	20.79	0.12	Xylopyranoside
10	1H-Indole-3-carboxaldehyde,	C_9H_7NO	145.158	21.39	0.05	L-Tryptophan metabolite
11	D-Arabinose	C5H10O5	150.129	23.33	2.37	Sugar
12	D-fructose	C6H12O6	180.155	24.66	7.72	Fructopyranose
13	α-D-Glucose	C6H12O6	180.155	24.90	13.1	Sugar
14	Glucitol	C6H14O6	182.171	25.44	49.29	Sugar alcohol
15	Benzoic acid, 3,4,5-trihydroxy-	C7H6O5	170.119	25.95	1.08	Gallic acid (phenolic acid)
16	ß-D-Glucopyranose	C6H12O6	180.155	26.29	3.54	Glucopyrasose derivative
17	Hexadecanoic acid, methyl ester	C17H34O2	270.450	27.51	0.44	Palmitic acid ester
18	Myo-Inositol	C6H12O6	180.155	27.96	0.78	Phytic acid
19	Lucenin 2	C27H30O16	610.521	29.10	0.11	Flavone
20	2H-1-Benzopyran-2-one, 4-hydroxy-	$C_9H_6O_3$	162.142	34.55	0.27	Coumarin
21	6,7-Dihydroxycoumarin-á-D-Glucopyranoside	C15H16O9	340.284	35.12	0.16	Coumarin glucoside
22	D-Fructose	C6H12O6	180.155	35.78	0.53	Sugar
23	α-D-Glucopyranose, 4-O-β-D-galactopyranosyl-	C12H22O11	342.296	36.18	0.41	Glucopyranose
24	Estra- 1,3,5(10)- trien-17ß- ol	C18H24O	256.382	37.37	0.15	Estradiol derivative
25	D-Glucose , 4-O-β-D-galactopyranosyl-	C12H22O11	342.296	38.65	0.05	Galactopyranose
26	Oleic acid, 3-(octadecyloxy)propyl ester	C39H76O3	593.034	41.16	0.27	Fatty acid
27	3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-4-chromen	C15H10O8	318.237	41.70	0.11	Flavonol (Myricetin)
28	á-D-Galactopyranoside, methyl	C7H14BO6	194.182	42.39	2.16	Galactopyranose
29	á-Sitosterol	C29H50O	414.706	42.70	0.20	Phytosterol
30	3,5,7- Trihydroxy-2-[3,4- diphenyl]- 4H-1-benzopyran- 4- one	C15H10O7	C15H10O7	44.20	0.75	Flavonol (Quercetin)
31	4H- 1-benzopyran-4 -one, 5,7- dihydroxy-2-[4-hydroxypheny l]	C16H12O6	300.266	46.48	0.54	Flavonol (kaempferol)
32	9-Octadecenoic acid, methyl ester	C19H36O2	296.487	47.62	0.01	Fatty acid (Oleic acid)
33	psi,psi-Carotene	C40H56	536.888	47.88	0.34	Terpenes (Lycopene)
34	4 H-1- Benzopyran-4-one, 2-(3, 4-dihydroxyphenyl)-5, 7- dihydroxy-	C15H10O6	286.239	49.21	0.48	Flavone (Luteolin)

the PGP treatment group at doses of 50, 100 and 250 g/mL, respectively. This demonstrates that cells with a greater PGP concentration accumulate more DNA. However, the opposite trend of % proportion of cells in S phase was observed in G_1 phase i.e., 77.49% (control), 76.46%, 70.92%, 32.57%, 65.25% at doses of 25 μM IM, and 50, 100, 250 μg/mL PGP respectively, suggesting the enhanced cell proliferation by stimulating the cell cycle from G_1 to S phase. However exceptionally, at 100 μg/mL PGP concentration shows a maximum reduction in G_1 phase (Fig. 4).

Inhibition of ROS formation

Cells incubated with H_2O_2 (stock solution with 10 μM) for about 24 h considerably decreases the chondrocytes number. The microscopic analysis from fluorescence microscope indicates that the fluorescence intensity was greatly reduced up to 65.1%, 60.0%, 44.14% with an increase in PGP

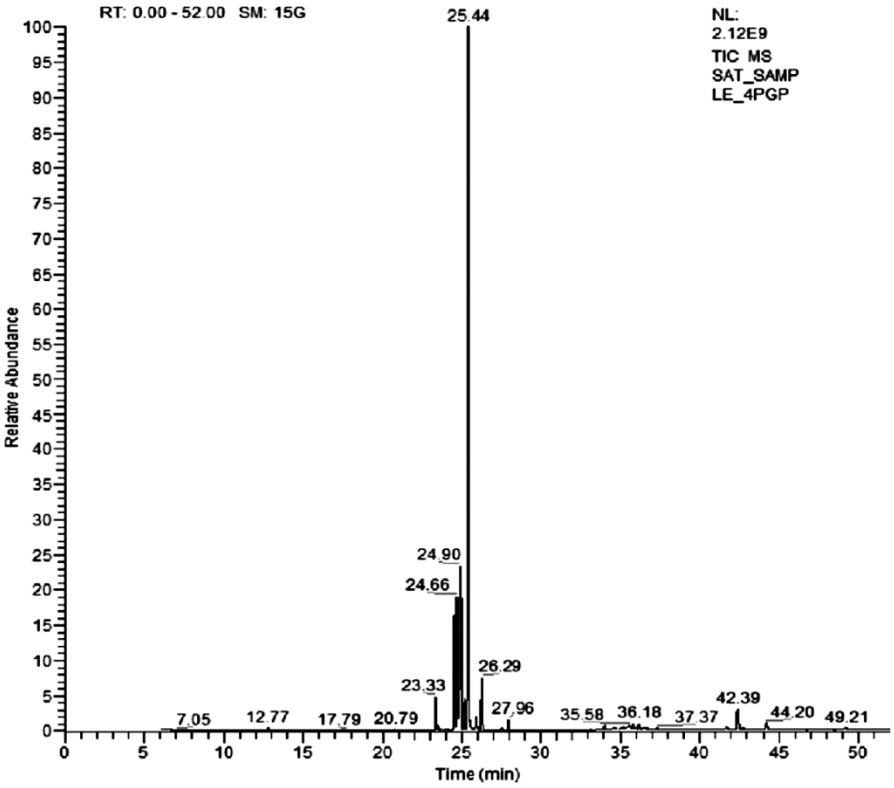

Fig. 1 — GC-MS Chromatogram of 95% ethanolic extract of *Punica granatum* peel (PGP).

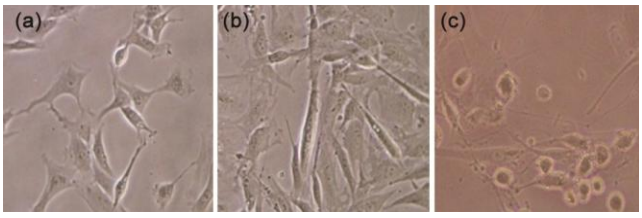

Fig. 2 — Photomicrographs representing the morphological observation of chondrocytes. (A) Passage 1 chondrocytes at 6 days of primary culture were polygonal and irregular in shape. (B) Passage 2 chondrocytes were more spindles in shape. (C) Passage 4 chondrocytes become oval, detaching partially. (Magnification: 20X; Scale bar: 0.1 mm).

concentration i.e., 50, 100 and 250 µg/mL, respectively compared to H_2O_2 control. The cells treated with IM showed approximately 10% reduction in ROS with a fluorescence intensity 70% as compared to H_2O_2 control in which the fluorescence intensity was observed to be 88.82%. The data of quantitative analysis also demonstrates the significant reduction in intracellular ROS production in all treatment groups and thus increases the cell viability (Fig. 5).

Inhibition of NO induced cell death validated from MTT assay

The release of NO is a crucial component in the pathophysiology of OA. We aimed to investigate

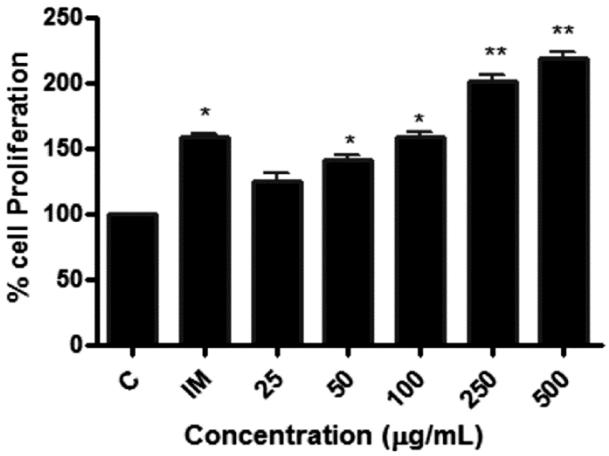

Fig. 3 — The graph demonstrates the effect of PGP on enhanced percent cell proliferation in a dose-dependent way. Data demonstrated mean and SEM value. The non-parametric test and one-way ANOVA showing the significance value **p≤0.001 and *p<0.01 *versus* control (C).

whether the PGP extract moderates cell death caused by NO generation. Treatment of chondrocytes with various doses of PGP before exposure to sodium nitroprusside (SNP) reduces cell mortality in a dose-dependent way (p<0.05%). The viability of cells reduces approximately 70% and reaches 30.4% in NO-control compared to control. However, the viability of cells increases in all

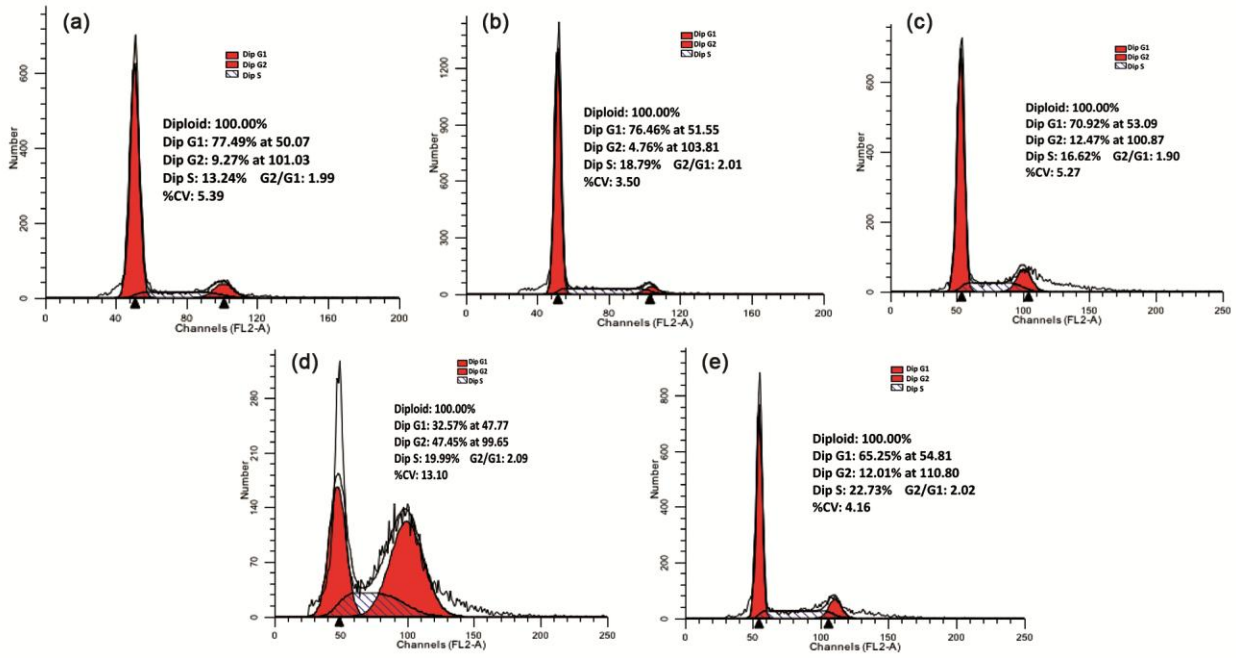

Fig. 4 — Effect of PGP extract on the cell proliferation of rat chondrocytes using Flow Cytometry. The seeded cells were remain starved for 24 h before treatment with PGP. The treated cells were collected and stained with PI followed by FACS analysis. (a) Control group without treatment (b) Treated with indomethacin, (c), (d), and (e) were treated with various concentrations of PGP extract i.e., 50, 100 and 250 µg/mL, respectively.

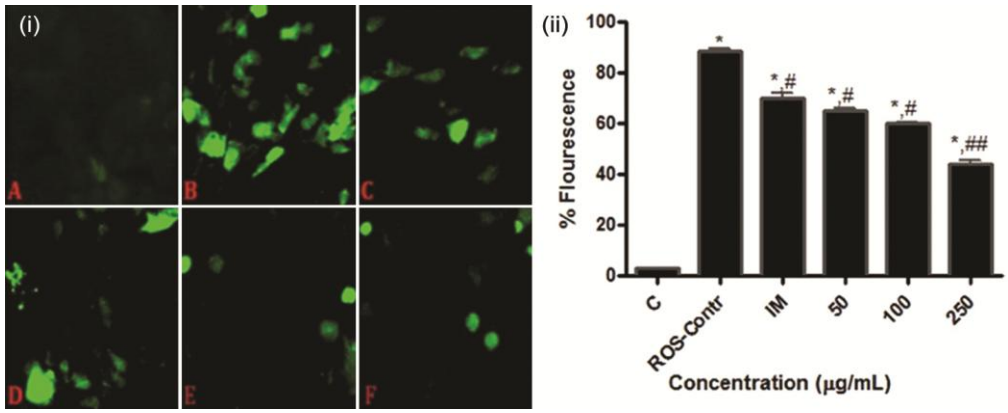

Fig. 5(i) — Photomicrographs of chondrocytes (10X) exposed to H_2O_2 solution for 24 h followed by treatment with IM and PGP extract. (A) Control group, (B) H_2O_2 control group, (C) IM, (D), (E), (F) at various concentration of PGP (50, 100 and 250 µg/mL). (ii) Graph shows the percent ROS production and calculated as number of cells positive for DCF staining to the total number of cells. All data were demonstrated as mean and SEM. Non-parametric test and one-way ANOVA represents the significance value *p<0.001 *versus* control and #p<0.05, ## p<0.01 *versus* H_2O_2 induced ROS control (ROS-contr).

the treatments groups up to 50.2%, 44.4%, 58.5%, 68.3% in IM, 50, 100, 250 µg/mL of PGP respectively, inhibits the apoptosis of cells due to the production of NO (Fig. 6).

Inhibition of NO induced nuclear condensation

It is cleared from photomicrograph (Fig. 7), that the cells exposed to only SNP (NO-control) without any pre-treatments showed maximum fluorescence with condensed and fragmented nuclei as compared to the control cells. The fluorescence intensity was reduced to 15.1% in cells pre-treated with IM, and 20.2%, 7.9% and 2.6 % in cells pre-treated with PGP at concentrations 50, 100, 250 µg/mL respectively, compared to NO-Control (31.5%). The significant ($p<0.05$) reduction of fluorescence was observed at 100 and 250 µg/mL of PGP concentration.

Inhibition of NO-induced apoptosis in the cells treated with IM and PGP

PI staining showed a reduction in the cell apoptosis rate when pre-treated with either IM or different doses of

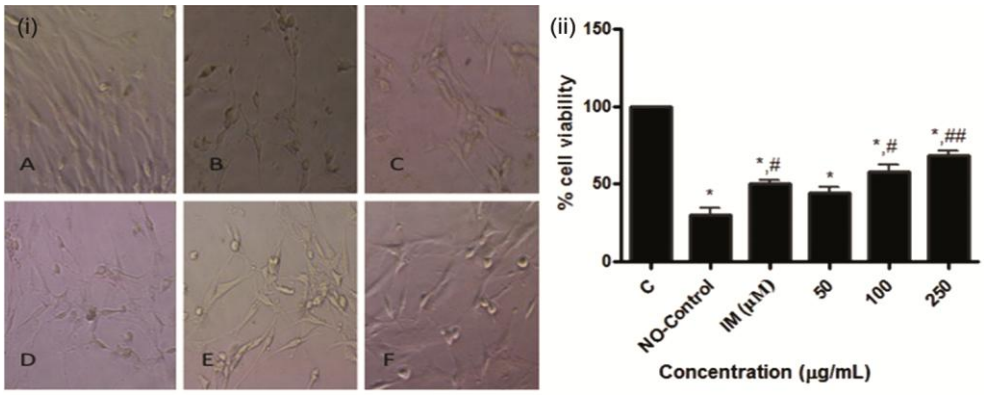

Fig. 6(i) — Photomicrographs of chondrocytes (Magnification = 20X with scale bar = 0.1 mm) pretreated with drug IM and PGP extract (24h) and further incubated with sodium nitroprusside for 24 h. (A) Control group, (B) NO-induced control group (NOC), (C) IM, (D), (E), (F) are doses at various concentration of extract (50,100, 250 μg/mL). (ii) The graph represents the quantitative analysis of the difference in cell viability. Mean and SEM were used to represent the data. Non-parametric test and one-way ANOVA showing the test of significance *p≤0.001 versus control and #p<0.05, ## p<0.01 versus the NOC (NO-Control).

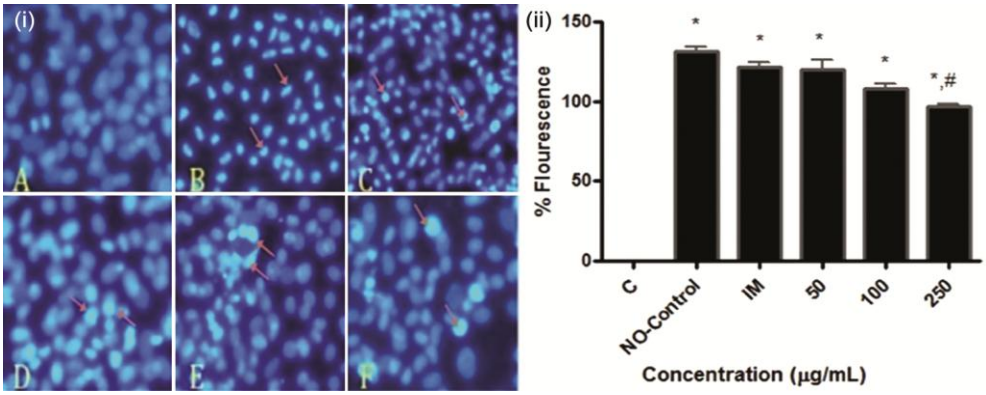

Fig. 7(i-ii) — Photomicrographs taken from fluorescence microscopy shows nuclear condensation in chondrocytes treated with PGP (50, 100, 250 μg/mL) concentrations. The Arrow shows fluorescence in highly fragmented DNA due to NO release. (ii) The graph represents % apoptotic cells, which were manually counted using fluorescence microscope in ten random fields, and represents % apoptotic cells. Values were calculated from independent experiments (n=3) and expressed as mean and SEM. *p< 0.001 compared to control and #p<0.05 compared to NO- induced control (NO- Contr). (Magnification = 20 X with scale bar=0.1 mm).

PGP (25, 50 and 100 μg/mL) before exposure to SNP. The fluorescence intensity of the stained cells correlates with the cells undergoing apoptosis. The maximum fluorescence was observed in NO-induced control without any pre-treatment. The intensity of fluorescence was slightly reduced in IM treated cells and at 50 μg/mL PGP treated cells. However, the intensity of fluorescence was considerably reduced in cells treated with PGP at concentrations 100 and 250 μg/mL (Fig. 8).

Discussion

OA is a multi-factorial disease with unknown etiology and its pathogenesis is influenced by oxidative stress that is produced by reactive oxygen species (ROS) that lead to the progression of the disease[28,29]. The commonly used NSAIDs do not modify the disease course and possess a significant risk in accelerating joint breakdown in OA[30,31]. The use of nutrition for the management of OA also lies in detriments that it can prevent the risk associated with that of NSAIDs. In this context, nutritional compounds containing bioactive components are advantageous. A wide range of natural compounds having antioxidant properties is widely used to relieve oxidative stress. These compounds are required for maintaining the chondrocyte proliferation and inhibition of apoptosis for the integrity of cartilage.

For this purpose, we selected PGP, a multifunctional natural fruit peel. From the chromatographic profile, we identified the bioactive components in PGP that mainly contained phytoconstituents including flavonols (quercetin, kaempferol, myricetin), the flavone (luteolin), phenolic acid (gallic acid) and pyrogallol, as well as, coumarin and its glycosides, ascorbic acid and

lycopene (terpene). The combination of these bioactive compounds has a therapeutic effect that might be beneficial in the proliferation of chondrocytes and delay the progression of OA by preventing apoptosis. Similarly, other plants like Cape gooseberry containing carotene, quercetin, myricetin and kaempferol exhibits antioxidant properties[32]. Apart bioactive phenolics, ascorbic acid, lycopene, a large number of sugars, D3 metabolite and polyunsaturated fatty acids are also present. The presence of these bioactive phytochemicals in PGP may be beneficial in maintaining the chondrocyte proliferation and inhibition of apoptosis for the integrity of cartilage. In a study on human chondrocytes, an antioxidant vitamin C decreases the rate of apoptosis and also decreases the H2O2-driven senescence of chondrocytes under oxidatively stressed condition[33].

This study was designed to understand the mechanistic insight of PGP and indomethacin (IM) in the chondrocytes proliferation. The efficiency of the extract on cell proliferation was directly evaluated using MTT assay and cell cycle analysis. The cell proliferation data suggest that there is increased proliferation of chondrocytes in a dose-dependent manner in PGP treated cells. It was significantly high as compared to both control and IM treated cells. In a study, the protective effect of *Capparis spinosa* containing the bioactive flavonoids like quercetin and kaempferol, on chondrocytes appeared to be greater than that elicited by indomethacin[34]. Also, our recent study has demonstrated that Punica granatum peel extract reduces OA in rats as compared to indomethacin[35]. The cell cycle analysis represents that the proportion of chondrocytes in the G_0/G_1 phase significantly lowers as the concentration of PGP increases. In contrast, the concentration of chondrocytes in S phase increases due to the increase or doubling of DNA content of chondrocytes with an increase in the concentration of PGP extract. This shows that PGP including IM precedes the cell-cycle progression from G_1 to S phase. The findings suggest that PGP induce chondrogenesis by increasing proliferation of primary chondrocyte and progression of cells from G_1 to S phase. Interestingly, a previous study on primary osteoblast also supports our findings of the proliferation activity of *P. granatum* extract was based on progression in DNA content in the S phase of the cell cycle in the cell[27].

ROS plays a significant role in the proliferation of cells and hence we attempt to develop an *in vitro* oxidative stress model by exposure of chondrocytes to an exogenous H_2O_2 for 24 h. It has been studied previously that H_2O_2 induces the chondrocyte death in a dose-dependent way and inhibits chondrocyte growth and the micromolar concentration of H_2O_2 produces potent oxidative stress DNA lesions[36-38]. The production of ROS due to H_2O_2 treatment is significantly reduced with an increase in the doses of PGP. Compared to ROS-Control, minimum inhibition of ROS was observed in IM treated cells and 50 µg/mL concentration of PGP extract. At 250 µg/mL concentration, maximum inhibition of ROS generation was observed. Therefore, PGP extract protects the chondrocyte from H_2O_2 induced ROS generation through its anti-inflammatory and antioxidant properties through a group of bioactive compounds present in PGP extract. This describes the inhibition of oxidative stress of chondrocytes that determines the chondroprotective effect of PGP in H_2O_2 induced ROS generation in chondrocyte cells. Studies have demonstrated the inhibitory activity of *Angelica sinensis* that reduces H_2O_2 induced cell apoptosis in rat as well as in human chondrocytes *via* exerting antioxidant, anti-inflammatory and anti-apoptotic properties[39,40]. In addition, anti-apoptotic properties of PGP were found in chondrocytes cells that were pretreated with PGP at different concentrations for 24 h followed by SNP treatment which generated NO-induced cell death[41]. The studies have suggested that NO cause oxidative stress and apoptosis of cell by enhancing the production of various inflammatory cytokines like TNF-α and IL-1β[42,43]. There are several studies that demonstrates

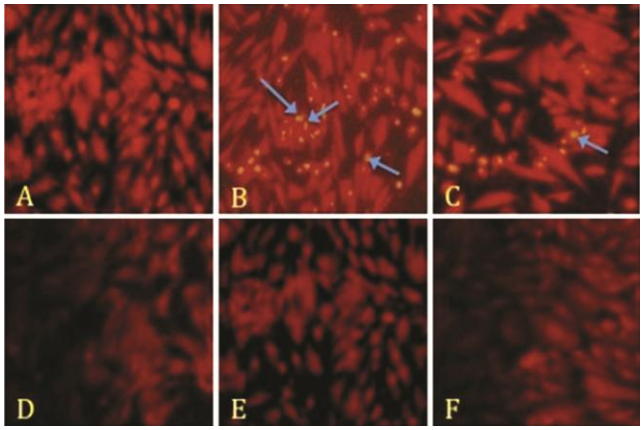

Fig. 8 — Effect of PGP extract on apoptosis. The cells marked by the arrow show apoptosis. (Magnification 20 X, Scale bar: 0.1 mm). (A) Control, (B) NO-induced control (NOC), (C) IM treated cells, (D), (E), (F) showed at different concentration (50, 100, 250 µg/mL) of PGP.

the correlation exist between nitric oxide (NO) production level and chondrocyte apoptosis[44,45]. The decreased cell viability in concentration-dependent manners has been observed with increased cellular NO levels in human chondrocytes exposed to SNP[41]. The consequences of SNP on chondrocytes was examined by parameters like cell viability using MTT assay and cell apoptosis through nuclear fragmentation and Hoechst- PI staining. It was observed from photomicrography images and statistical data that PGP induces proliferation and reduces the apoptosis of chondrocytes in a dose-dependent mode in NO-induced *in vitro* apoptotic test system. The maximum inhibition of apoptosis occurs at 250 μg/mL of concentration where the PGP reduces the apoptosis and the cell viability reaches up to 68.30%. The study demonstrates PGP display chondroprotective role against NO-induced cell death might be via inhibiting various pro-inflammatory cytokines involving chondrocyte death. The previous study showed that Sumac leaves also inhibit the production of ROS and NO that is stimulated by pro-inflammatory cytokine in human chondrocyte cells[6]. The therapeutic properties of PGP are due to the occurrence of numerous bioactive phytochemicals as flavonols (quercetin, kaempferol, myricetin), the flavone (luteolin), phenolic acid (gallic acid) and pyrogallol, as well as, coumarin and its glycosides, ascorbic acid and lycopene (terpene). The flavonoids have anti-oxidant properties owing to its native origin and has high free radicals scavenging capabilities[46]. Our study also demonstrates that these phytochemicals induce the proliferation of chondrocytes through the progression of cells from G1 to S phase in the cell cycle as well as inhibits NO and H_2O_2 induced apoptosis in chondrocytes. Further studies are needed to validate our findings at *in vivo* level.

Conclusion

Our findings reveal that PGP extract is enriched in bioactive phenolic (flavone, flavonoids), phenolic acids, lycopene and ascorbic acid, which together comprise an excellent source of antioxidant chemicals that block the key mediator and causes chondrocyte degradation. The proliferation of chondrocytes is stimulated by PGP which results in chondrocytes proliferation through progressing cell cycle from G_1 to S phase. In addition, the results show that PGP has anti-apoptotic properties via inhibiting ROS generation and NO-induced cell death.

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Conflict of Interest

All authors have none to declare.

Authors' Contributions

MA and NS conceived the study and designed the experiment. NS, SS, JR and VR performed the experiments. NS, SS, VR, AJ, MSK and MA analyzed data and interpreted results and co-wrote and edited the manuscript. SS, MG, MSK and MA provide critical inputs and revised the manuscript. All authors reviewed the manuscript.

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